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1       **Glucose starvation and hypoxia, but not the saturated fatty acid palmitic acid or**  
2       **cholesterol, activate the unfolded protein response in 3T3-F442A and 3T3-L1 adipocytes**

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13      response

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## 16   **Abstract**

17   Obesity is associated with endoplasmic reticulum (ER) stress and activation of the unfolded  
18   protein response (UPR) in adipose tissue. In this study we identify physiological triggers of  
19   ER stress and of the UPR in adipocytes *in vitro*. We show that two markers of adipose tissue  
20   remodelling in obesity, glucose starvation and hypoxia, cause ER stress in 3T3-F442A and  
21   3T3-L1 adipocytes. Both conditions induced molecular markers of the IRE1 $\alpha$  and PERK  
22   branches of the UPR, such as splicing of *XBPI* mRNA and CHOP, as well as transcription of  
23   the ER stress responsive gene *BiP*. Hypoxia also induced an increase in phosphorylation of  
24   the PERK substrate eIF2 $\alpha$ . By contrast, physiological triggers of ER stress in many other cell  
25   types, such as the saturated fatty acid palmitic acid, cholesterol, or several inflammatory  
26   cytokines including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, do not cause ER stress in 3T3-F442A and 3T3-  
27   L1 adipocytes. Our data suggest that physiological changes associated with remodelling of  
28   adipose tissue in obesity, such as hypoxia and glucose starvation, are more likely physiologic  
29   ER stressors of adipocytes than the lipid overload or hyperinsulinemia associated with  
30   obesity.

## 31   **Introduction**

32   Obesity is the leading risk factor for type 2 diabetes, cardiovascular disease, and  
33   hypertension.<sup>1,2</sup> Obesity affects the homeostasis of the whole body but mainly the liver and  
34   the adipose tissue, and is characterized by low grade inflammation, hyperlipidemia, and  
35   insulin resistance in surrounding and peripheral tissues.<sup>1,2</sup> Adipose tissue is exposed to  
36   several stresses in obesity, including inflammation, hypoxia, and endoplasmic reticulum (ER)  
37   stress.<sup>3</sup> Limited angiogenesis, adipocyte hypertrophy and hyperplasia cause hypoxia in obese  
38   adipose tissue.<sup>4</sup> Secretion of MCP-1 by dysfunctional adipocytes attracts circulating  
39   monocytes into adipose tissue,<sup>5,6</sup> while a change in the adipokine profile, including decreased  
40   adiponectin and increased leptin secretion,<sup>5</sup> may contribute to the replacement of adipose

41 tissue resident alternatively activated (M2) macrophages with classically activated (M1)  
42 macrophages.<sup>6</sup> While physiologic causes of inflammation and hypoxia in adipose tissue have  
43 been characterised, little is known about the physiologic triggers of ER stress in obese  
44 adipose tissue. At the molecular level, ER stress is caused by the build-up of misfolded  
45 proteins in the ER and activation of a signalling network called the unfolded protein response  
46 (UPR).<sup>7</sup> The UPR attempts to restore ER homeostasis by inducing expression of genes  
47 encoding molecular chaperones and protein foldases, lipid biosynthetic enzymes, and proteins  
48 involved in ER-associated protein degradation. If the ER stress cannot be resolved, the UPR  
49 promotes apoptosis. ER stress also plays key roles in both inflammation and insulin  
50 resistance in obesity and type 2 diabetes.<sup>8, 9</sup>

51 In mammalian cells, three UPR signalling cascades are initiated by the ER  
52 transmembrane proteins PERK, IRE1 $\alpha$ , and ATF6. Phosphorylation of the translation  
53 initiation factor eIF2 $\alpha$  by the protein kinase PERK inhibits general translation, but also  
54 stimulates translation of mRNAs harbouring several short upstream open reading frames in  
55 their 5' untranslated regions. This mechanism of translational activation results in induction  
56 of the transcription factors ATF4 and C/EBP homologous protein (CHOP).<sup>10, 11</sup> CHOP  
57 reactivates protein synthesis and oxidation in the ER.<sup>12</sup> IRE1 $\alpha$  up-regulates ER chaperone  
58 genes and genes involved in ER-associated protein degradation via endoribonuclease domain-  
59 induced splicing of X-box protein 1 (XBP1) mRNA.<sup>13, 14</sup> The transcription factor ATF6  
60 translocates to the nucleus after proteolytic release from the Golgi membrane by the Golgi  
61 proteases S1P and S2P<sup>15</sup> and induces expression of genes encoding ER resident molecular  
62 chaperones and proteins functioning in ER-associated protein degradation.<sup>16, 17</sup> Upon  
63 prolonged or irremediable ER stress the UPR induces apoptosis via activation of JNK<sup>18</sup> by  
64 IRE1 $\alpha$  and TRB3 by CHOP.<sup>19</sup>

65 The physiological factors leading to ER stress and activation of the UPR in obese  
 66 adipocytes are not well characterized. For several other cell types, including hepatocytes,  
 67 pancreatic  $\beta$  cells, and macrophages physiologic ER stressors have been reported. Saturated  
 68 fatty acids (SFAs) or cholesterol loading induce an UPR in several cell types such as  
 69 hepatocytes,<sup>20, 21</sup> pancreatic  $\beta$  cells,<sup>22</sup> macrophages,<sup>23</sup> and preadipocytes.<sup>24</sup> Inflammatory  
 70 cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , which are secreted by stressed adipocytes or  
 71 macrophages recruited into inflamed adipose tissue,<sup>25</sup> elicit an ER stress response in L929  
 72 myoblast cells and hepatocytes.<sup>26, 27</sup> Glucose starvation is the earliest identified physiological  
 73 ER stressor,<sup>28, 29</sup> while the hypoxic environment of tumours induces an UPR in tumour  
 74 cells.<sup>30-32</sup>

75 The purpose of this study was to identify obesity-related physiological inducers of ER  
 76 stress and the UPR in adipocytes by exposing *in vitro* differentiated 3T3-F442A adipocytes to  
 77 several physiologic ER stressors, including the SFA palmitic acid, cholesterol, inflammatory  
 78 cytokines, glucose starvation, and hypoxia. We report that potent physiologic ER stressors in  
 79 other cell types, such as palmitic acid, cholesterol, or the inflammatory cytokines TNF- $\alpha$ , IL-  
 80 1 $\beta$ , and IL-6, do not induce an ER stress response in *in vitro* differentiated 3T3-F442A or  
 81 3T3-L1 adipocytes. Glucose starvation and hypoxia, however, induce markers of ER stress,  
 82 such as splicing of *XBPI* mRNA, transcriptional activation of ER stress responsive genes  
 83 including *BiP*, and *ERDJ4*, CHOP and phosphorylation of eIF2 $\alpha$ . Our results suggest that  
 84 hypoxia and glucose starvation are likely physiologic ER stressors for adipocytes *in vivo*.

## 85 **Results**

### 86 *Palmitate does not induce ER stress in adipocytes*

87 To identify which obesity-related physiological factors trigger the UPR in adipocytes we  
 88 exposed *in vitro* differentiated 3T3-F442A and 3T3-L1 adipocytes to several compounds  
 89 whose plasma levels are elevated in obesity,<sup>33-39</sup> including palmitic acid, cholesterol, and the

inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. 3T3-F442A adipocytes were chosen because these cells form normal adipose tissue without the addition of exogenous inducers when implanted subcutaneously into athymic mice.<sup>40, 41</sup> 3T3-L1 adipocytes were included to provide a second source of adipocytes. Both cell lines were differentiated for 12 d and the percentage of cells with an increased lipid content determined by flow cytometry with the fluorescent lipid probe Nile Red.<sup>42</sup> Flow cytometry revealed a mean fluorescence increase of  $3.2 \pm 0.2$  fold upon differentiation of 3T3-L1 cells (Fig. 1A). In differentiated 3T3-F442A cells two populations with  $2.9 \pm 0.1$  fold and  $25 \pm 2$  fold increases in Nile Red fluorescence were distinguishable (Fig. 1B). An  $\sim 3$  fold increase in Nile Red fluorescence in differentiated 3T3-L1 adipocytes and the larger population of differentiated 3T3-F442A adipocytes is in good agreement with previously published increases in Nile Red fluorescence during differentiation of human adipocytes<sup>43</sup> and adipogenic differentiation of the murine embryonic stem cell line CGR8.<sup>44</sup> Quantitation of the histograms for the Nile Red fluorescence by constructing the probability distribution for the increase in Nile Red fluorescence upon differentiation and the constraint that the Nile Red fluorescence of adipocytes has to be greater by at least two standard deviations of the mean Nile Red fluorescence of undifferentiated cells than the Nile Red fluorescence of undifferentiated cells reveals that  $72 \pm 3$  % of the 3T3-L1 and  $80 \pm 1$  % of the 3T3-F442A cells acquired a lipid-laden phenotype. These degrees of differentiation are comparable to previously published data.<sup>45</sup>

The granularity of cells increases during differentiation into adipocytes because of the accumulation of lipid droplets.<sup>46</sup> This increase in granularity is reflected by an increase in the side scatter of the exciting laser beam<sup>47</sup> and is also seen after differentiation of both 3T3-L1 and 3T3-F442A cells for 12 d (Fig. 1C-D). The side scatter of the highly fluorescent 3T3-F442A adipocyte population ( $\geq 300$  A.U. in Fig. 1B) is significantly higher than the side scatter of the weaker fluorescent population ( $< 300$  A. U., Fig. S2), suggesting that the highly

fluorescent cells contain more lipid droplets than the weaker fluorescing population. Forward scatter, which is affected by cell size and shape,<sup>47</sup> decreases in 3T3-L1 cells and becomes more heterogeneous in 3T3-F442A cells (Fig. 1E-F). Taken together, these data suggest that the majority of the 3T3-L1 and 3T3-F442A cells have acquired a lipid-laden phenotype 12 d after initiation of adipogenic differentiation.

To determine whether palmitic acid causes ER stress in adipocytes *in vitro*, 3T3-L1 and 3T3-F442A adipocytes were incubated with different concentrations (0–1 mM) of palmitate complexed to fatty acid-free bovine serum albumin (BSA) for up to 48 h. The activity of the PERK branch of the UPR was assessed by Western blotting for CHOP, while activation of IRE1 $\alpha$  was monitored by measuring splicing of *XBPI* mRNA. Exposure of adipocytes to up to 1 mM palmitate for 48 h did not elevate CHOP levels (Fig. 2A-B), induce detectable levels of *XBPI* splicing (Fig. 2C-D, S3-7), or elevate mRNA levels for the ER stress responsive genes *BiP* (Fig. 3A-B), *CHOP* (Fig. 3C-D), or *ERDJ4* (Fig. 3E-F) especially when compared to the large increases in mRNA levels of these genes and CHOP protein levels in thapsigargin-treated adipocytes (Figs. 2A-B and 3). Treatment with palmitate complexed to BSA for 8 or 24 h did also not induce *XBPI* splicing in 3T3-F442A adipocytes (Figs. S5-7). Palmitate did also not affect the viability of 3T3-F442A adipocytes over a period of up to 48 h, while incubation with 1  $\mu$ M thapsigargin, which causes ER stress by depleting ER luminal Ca<sup>2+</sup> stores,<sup>48</sup> for 48 h decreased viability by ~37% (Fig. 2E). Palmitate did also not inhibit insulin-stimulated AKT serine 473 phosphorylation in 3T3-F442A adipocytes (Fig. 4A), which is consistent with several other reports.<sup>49-56</sup> To validate that our BSA-palmitate complexes induce ER stress, we characterised *XBPI* splicing in undifferentiated preadipocytes exposed to palmitate complexed to BSA. Exposure of preadipocytes to palmitate complexed to BSA induces *XBPI* splicing in these cells.<sup>24</sup> Indeed, palmitate induced *XBPI* splicing in undifferentiated preadipocytes (Figs. 2F and S8) and also inhibited

insulin action in these cells (Fig. 4B). Collectively, these results show that the SFA palmitic acid does not induce ER stress in adipocytes.

#### *Cholesterol does not induce an UPR in adipocytes*

To characterize whether cholesterol elicits ER stress in adipocytes we exposed differentiated 3T3-F442A and 3T3-L1 adipocytes to 100  $\mu$ g/ml AcLDL for 48 h. AcLDL did not elevate CHOP levels (Fig. 5A-B), induce *XBPI* splicing (Figs. 5C-D and S9A-B), or elevate *BiP* or *CHOP* mRNA levels (Fig. 6). We, therefore, repeated these experiments in the presence of the ACAT inhibitor TMP-153 to inhibit cholesterol esterification and to elevate intracellular free cholesterol levels. After 24 h no changes in expression of CHOP or in *XBPI* splicing were observed (data not shown). 48 h of treatment with AcLDL and TMP-153 did not increase CHOP protein levels (Fig. 5A-B), induce *XBPI* splicing (Fig. 5C-D), or elevate the mRNA levels for *BiP* (Fig. 6A-B) or *CHOP* (Fig. 6C-D). To validate that AcLDL can, in principle, activate the UPR, we repeated these experiments with *in vitro* differentiated THP-1 macrophages which are known to develop ER stress in response to cholesterol overloading.<sup>57</sup> In differentiated THP-1 macrophages AcLDL induced *XBPI* splicing both in the presence and absence of TMP-153 (Figs. 5E and S9C). Treatment of THP-1 macrophages with TMP-153 alone also increased *XBPI* splicing ~2.6 fold (Figs. 5E and S9C). These results suggest that exposure of adipocytes to AcLDL does not cause ER stress.

#### *Proinflammatory cytokines do not induce ER stress in adipocytes*

To study whether inflammatory cytokines induce ER stress in adipocytes we exposed differentiated 3T3-F442A adipocytes to various concentrations of TNF- $\alpha$ , IL-6, or IL-1 $\beta$  for up to 24 h. Incubation of adipocytes with increasing concentrations of TNF- $\alpha$  for 24 h did not affect the viability of these cells (Fig. 7A), but also failed to induce *XBPI* splicing (Figs. 7B and S10). Various concentrations of IL-6 and IL-1 $\beta$  also failed to induce *XBPI* splicing over a period of 24 h (Figs. 7D-E and S11-12). To validate that the cytokines possess biological



activity we characterized activation of the MAPK kinase JNK in preadipocytes. All three cytokines stimulated phosphorylation of JNK (Fig. 7C and F), thus providing evidence that the cytokine preparations we utilised possess biological activity. Taken together, these data suggest that the inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  do not cause ER stress in adipocytes.

#### *Glucose starvation induces ER stress in adipocytes*

Prolonged exposure of cells to glucose concentrations of <0.2 g/l induces the ER resident chaperones BiP and GRP94,<sup>28, 58</sup> whose expression is controlled by XBP1 and ATF6. To characterize whether glucose starvation, which may be caused by the poor vascularization of the expanding adipose tissue in obesity, can induce ER stress in adipocytes, we maintained *in vitro* differentiated 3T3-F442A and 3T3-L1 adipocytes for up to 24 h in serum free medium supplemented with 2 mM L-glutamine but completely lacking glucose. Glutaminolysis serves as an energy source in this medium.<sup>59, 60</sup> Glucose starvation for 24 h induced CHOP potently in both 3T3-F442A and 3T3-L1 adipocytes (Fig. 8A-B). *XBP1* splicing peaked 12 h after induction of glucose starvation (Fig. S13A) and remained elevated for the next 36 h in 3T3-F442A-adipocytes (Figs. 8C-D and S13B). 24 h of glucose starvation also induced *XBP1* splicing in 3T3-L1 adipocytes and elevated the steady-state mRNA levels of *CHOP*, *BiP*, and *ERDJ4*, and, to a lesser extent, *EDEMI* and *VEGFA* mRNAs in 3T3-F442A adipocytes (Fig. 8E). Thus, glucose starvation causes ER stress in adipocytes which coincides with increased expression of the pro-angiogenic factor *VEGFA*.

#### *Hypoxia causes ER stress in adipocytes*

We characterized whether hypoxia causes ER stress in *in vitro* differentiated 3T3-F442A adipocytes, because hypoxia is another physiological alteration in poorly vascularized obese adipose tissue.<sup>3</sup> *In vitro* differentiated 3T3-F442A adipocytes were cultured in 0.5% O<sub>2</sub> for up to 8 h before protein extraction and characterisation of ER stress markers and the hypoxia

marker HIF1 $\alpha$ <sup>61</sup> by Western blotting. Hypoxia increased HIF1 $\alpha$  levels within 2 h (Fig. 9A-B) and also led to an increase in eIF2 $\alpha$  phosphorylation (Fig. 9A-B), *XBPI* splicing (Fig. 9C-D), and *BiP* mRNA levels (Fig. 9E-F). The increases in *XBPI* splicing, *BiP* mRNA levels, and eIF2 $\alpha$  phosphorylation, once manifested, persisted throughout the time course of the experiment. Collectively, these data show that hypoxia induces ER stress in adipocytes.

## Discussion

We present evidence that glucose starvation and hypoxia (Figs. 8 and 9), but not palmitate (Figs. 2, 3 and S3-7), cholesterol (Figs. 5, 6, and S9), or several inflammatory cytokines (Fig. 7 and S10-12) cause ER stress in two *in vitro* adipocyte models, 3T3-F442A and 3T3-L1. These data suggest that the poor vascularization of adipose tissue in obesity causes ER stress in adipocytes, because adipose tissue expansion in obesity leads to formation of poorly vascularized, hypoxic areas.<sup>3, 4</sup> Glucose starvation may contribute to the adverse effects of hypoxia on adipose tissue, because obese adipocytes reach diameters that are comparable to the maximum distance of diffusive glucose supply from a blood vessel.<sup>62-64</sup> The large overlap of the effects of hypoxia and ER stress on adipose tissue, including inflammation,<sup>4</sup> insulin resistance,<sup>65</sup> changes in adiponectin secretion,<sup>66</sup> and increased angiogenesis,<sup>67-69</sup> suggests that ER stress may contribute to or mediate the effects of hypoxia on adipocytes.

Our work also suggests that palmitate, cholesterol, and inflammatory cytokines do not elicit an ER stress response in adipocytes. The mRNA expression for two ER stress sensors, IRE1 $\alpha$  and PERK, is similar in preadipocytes and adipocytes (Fig. S14), which suggests that increased basal activity of these ER stress signalling pathways cannot explain the protection of adipocytes from palmitate- or cholesterol-induced ER stress. A dominant feature of adipocyte differentiation is the induction of nearly all enzymes of fatty acid and triacylglycerol synthesis, including stearyl-CoA desaturases and diacylglycerol acyltransferases.<sup>70, 71</sup> Hence, adipocytes may be protected from palmitate-induced ER stress

because of their greatly increased ability to dispose of excess palmitate in their triacylglycerol pool.<sup>72</sup> The expansion of the triacylglycerol pool will also increase the storage capacity of adipocytes for cholesterol<sup>73, 74</sup> and thus may explain why cholesterol does not induce ER stress in adipocytes. Increased cholesterol efflux due to increased expression of the cholesterol transporter ABCA1<sup>75, 76</sup> may also contribute to this cholesterol resistance. Induction of several antioxidant enzymes<sup>77-79</sup> and increased NADPH generation<sup>80</sup> may protect adipocytes against ER stress caused by inflammatory cytokines, because these cytokines cause ER stress via production of reactive oxygen species.<sup>27, 81, 82</sup>

Our conclusions differ from conclusions drawn in other studies, which suggest that TNF- $\alpha$ ,<sup>83</sup> free fatty acids,<sup>84-87</sup> and cholesterol<sup>88</sup> induce ER stress in adipocytes *in vitro*. Koh *et al.*<sup>83</sup> and Jeon *et al.*<sup>85</sup> have reported that TNF- $\alpha$  and palmitate elevate phosphorylation of eIF2 $\alpha$ , induce *ATF3* mRNA and activate JNK in 3T3-L1 adipocytes and, on the basis of these changes, concluded that TNF- $\alpha$  and palmitate cause ER stress in adipocytes. eIF2 $\alpha$  phosphorylation and the increase in *ATF3* mRNA downstream of eIF2 $\alpha$  phosphorylation are controlled by four protein kinases<sup>89</sup> of which only PERK directly responds to ER stress.<sup>90</sup> JNK is activated by many stresses.<sup>91</sup> The absence of an increase in *XBPI* splicing (Figs. 2C-D, 7B, S3-7, and S10), which is a more specific marker for ER stress, suggests that other stresses are responsible for the increase in the stress markers monitored by Koh *et al.*<sup>83</sup> and Jeon *et al.*<sup>85</sup> Kawasaki *et al.*<sup>86</sup> have reported that exposure of 3T3-L1 adipocytes to 50  $\mu$ g/ml of a free fatty acid mixture derived from human serum induces *XBPI* splicing, *ATF4*, *BiP*, *CHOP*, *EDEM*, *ERDJ4*, and *PDI* mRNAs. Palmitic acid is considered to be the fatty acid with the greatest potential for cell injury,<sup>92</sup> but elicits ER stress, insulin resistance, or cell injury only at much higher concentrations in several cell types (Fig. 2F and refs. 21, 22, 24, 93) and does not induce ER stress in 3T3-F442A or 3T3-L1 adipocytes (Figs. 2, 3, and S3-7). Therefore, compounds other than the SFAs present in the fatty acid mixture used by

Kawasaki *et al.*<sup>86</sup> seem to be causing ER stress in adipocytes. Jiao *et al.*<sup>87</sup> reported that a mixture of lauric, myristic, oleic, linoleic, and arachidonic acids induces ER stress and potentially inhibits insulin-stimulated AKT serine 473 and threonine 308 phosphorylation in *in vitro* differentiated 3T3-L1 adipocytes. These results contradict not only our observations (Figs. 2, 3, and S3-7) but also several other papers which have reported that the unsaturated fatty acids oleic and linoleic acid protect cells from the negative effects of SFAs,<sup>94-100</sup> that the medium-chain fatty acids lauric and myristic acid do not induce insulin resistance,<sup>52</sup> and that palmitate does not affect insulin-stimulated AKT phosphorylation in adipocytes.<sup>49-56</sup> Chen *et al.*<sup>88</sup> reported that oxLDL induces BiP and CHOP in 3T3-L1 adipocytes and suggested that intracellular cholesterol overload may be partially responsible for this ER stress response. Both AcLDL and oxLDL are taken up via the scavenger receptor A by adipocytes.<sup>101</sup> We have not observed activation of *XBPI* splicing in 3T3-F442A or 3T3-L1 adipocytes exposed to AcLDL (Figs. 5 and S9), which suggests that an oxidized lipid or oxidized protein component of oxLDL,<sup>102</sup> but not cholesterol, induces ER stress in adipocytes *in vitro*.

In conclusion, our work shows that glucose and oxygen deprivation cause ER stress in adipocytes *in vitro*. In obesity, the rapid expansion of the adipose tissue rather than elevated SFAs, cholesterol, or proinflammatory cytokine levels, may be responsible for ER stress in adipocytes. Future work should address whether improved vascularization of obese adipose tissue, either through genetic or pharmacologic means, can mitigate ER stress in this tissue.

## Materials and Methods

**Antibodies and reagents.** Antibodies against AKT (cat. no. 4691), phosphoserine 473-AKT (cat. no. 4060), CHOP (cat. no. 2895), phospho-JNK (cat. no. 4668), JNK (cat. no. 9258), and phospho-eIF2 $\alpha$  (cat. no. 9721) were purchased from Cell Signaling Technology Inc. The anti-eIF2 $\alpha$  antibody (cat. no. sc-11386) was purchased from Santa Cruz Biotechnology Inc., the anti-HIF1 $\alpha$  antibody (cat. no. AF1935) from R&D Systems, the anti-GAPDH antibody (cat.

no. G8795) and the monoclonal anti- $\beta$ -actin antibody (cat. no. A2228) from Sigma-Aldrich. The goat anti-rabbit-IgG (H+L)-horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 7074S) was bought from Cell Signaling Technology Inc. The goat anti-mouse IgG (H+L)-HRP-conjugated antibody (cat. no. 31432) and the mouse anti-goat IgG (H+L)-HRP-conjugated antibody (cat. no. 31400) were purchased from Thermo Fisher Scientific. Thapsigargin, dexamethasone (cat. no. D4902), 3-isobutyl-1-methylxanthine (IBMX, cat. no. I5879), insulin (cat. no. I0516), palmitic acid (cat. no. P5585), fatty acid free bovine serum albumin (BSA, cat. no. A3803), BSA (cat. no. A2153), and thiazolyl blue tetrazolium bromide (MTT, cat. no. M5655), and 9-diethylamino-5*H*-benzo[ $\alpha$ ]phenoxazine-5-one (nile red, cat. no. N3013) were purchased from Sigma-Aldrich. TMP-153 was purchased from Enzo Life Sciences. Native human acetylated LDL (cat. no. 5685-3404) was purchased from AbD Serotec, IL-1 $\beta$  (cat. no. RIL1BI) from Thermo Fisher Scientific, IL-6 (cat. no. PHC0066) from Life Technologies, and human TNF- $\alpha$  (cat. no. 8902) from Cell Signaling Technology Inc.

**Cell culture.** 3T3-L1 murine preadipocytes<sup>103</sup> were obtained from the ATCC and were maintained as subconfluent cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine and 10% (v/v) bovine calf serum. 3T3-F442A murine preadipocytes<sup>104</sup> were maintained in DMEM supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine and 10% (v/v) foetal bovine serum (FBS). For differentiation,<sup>45</sup> both cell lines were grown to confluence. Two days post-confluency, differentiation was induced by addition of 1  $\mu$ g/ml insulin, 0.5 mM IBMX, and 0.25  $\mu$ M dexamethasone to the medium. The cells were maintained in this medium for three days and then for two more days in medium containing 1  $\mu$ g/ml insulin. After five days of differentiation insulin was omitted from the medium and the cells were maintained for another seven days. In all experiments both 3T3-F442A and 3T3-L1 adipocytes were used 12

d after induction of differentiation. The THP-1 human monocytic leukaemia cell line<sup>105</sup> was maintained in RPMI 1640 medium containing 10% (v/v) foetal bovine serum (FBS) and 2 mM L-glutamine. The cells were differentiated into macrophages by incubation with 50 nM phorbol-12-myristate 13-acetate (PMA) for 3 d, followed by incubation for 1 d without PMA.<sup>106</sup> Before addition of AcLDL or TMP-153 the cells were serum-starved for 7 h.

**Flow cytometry.** Cells were stained with Nile red and analysed by flow cytometry essentially as described before.<sup>42</sup> In brief, cells were trypsinised, washed once with DMEM supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine and 10% (v/v) bovine calf serum, and then with phosphate-buffered saline (PBS, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 27 mM KCl, 137 mM NaCl, pH 7.4), stained for 5 min with 100 ng/ml Nile red in PBS, washed once with PBS and immediately analysed by flow cytometry on a BD FACSCalibur Flow Cytometer (BD Biosciences) at a LO flow rate. For each sample ~50,000 gated events were collected. Nile red fluorescence was excited at 488 nm and its fluorescence emission collected using the FL-1 (530/30 nm) band pass filter set. The instrument settings for 3T3-L1 cells were FSC – E-1 (lin, Amp gain = 4.50), SSC – 326 V (lin, Amp gain = 1.00), and FL1 – 275 V (log, Amp gain = 1.00), and for 3T3-F442A cells FSC – E-1 (lin, Amp gain = 4.50), SSC – 280 V (lin, Amp gain = 1.00), and FL1 – 275 V (log, Amp gain = 1.00). No thresholds were applied. Data were analysed in WinMDI 2.9 and graphs prepared in GraphPad Prism 6.04 (GraphPad Software). Three biological replicates were analysed for each sample and results are represented as the average and standard error of these three repeats.

**Cell viability** was determined using the MTT assay.<sup>107</sup> In short, after TNF- $\alpha$  or palmitate treatment cells were incubated for 4 h at 37°C with 0.5 g/l MTT in phenol-red free DMEM containing 4.5 g/l D-glucose, and 2 mM L-glutamine or 2% (w/v) BSA, respectively. Insoluble formazan crystals were dissolved for 15 min in isopropanol containing 4 mM HCl and 0.1% (v/v) Nonidet P-40. The absorbance of the formazan solution was read at a

wavelength of 590 nm and a reference wavelength of 620 nm and the formazan absorbance expressed as the ratio of the absorbance at 590 nm to the absorbance at 620 nm.

**Palmitate treatment.** *In vitro* differentiated 3T3-F442A adipocytes were serum-starved overnight in DMEM containing 4.5 g/l D-glucose, and 2 mM L-glutamine and then incubated in serum-free medium containing 2% (w/v) fatty acid-free BSA and 0.05-1 mM palmitic acid. These palmitate concentrations are in the physiological range reported for rodents and humans.<sup>108</sup> Palmitic acid was complexed to fatty acid-free BSA as follows. In brief, palmitic acid was dissolved in ethanol and diluted 1:100 in DMEM containing 4.5 g/l D-glucose and 2% (w/v) fatty acid-free BSA before addition to the cells. Control cells received ethanol diluted 1:100 into DMEM containing 4.5 g/l D-glucose and 2% (w/v) fatty acid-free BSA.<sup>109</sup>

**Cholesterol and cytokine treatments.** *In vitro* differentiated adipocytes were incubated in DMEM containing 4.5 g/l D-glucose, 2 mM L-glutamine, and 100 µg/ml human acetylated LDL (AcLDL) in the presence or absence of the acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor TMP-153 at a final concentration of 0.6 µM. The cells were incubated with cytokines in serum-free medium.

**D-Glucose starvation experiments** were performed by incubating the cells for the indicated times in D-glucose-free DMEM supplemented with 2 mM L-glutamine. Control cells ('+ D-glucose') were incubated for the same time in DMEM containing 4.5 g/l D-glucose and 2 mM L-glutamine.

**Hypoxia experiments** were performed using a Billups-Rotenberg hypoxia chamber. A pre-analysed gas mixture of 0.5% (v/v) O<sub>2</sub>, 5% (v/v) CO<sub>2</sub> and nitrogen (BOC Industrial Gases) was flushed through the chamber at a flow rate of 25 l/min for 5 min to completely replace air inside the chamber with the gas mixture. The hypoxia chamber was incubated at 37°C for the indicated times. Cells were rapidly harvested and lysed at 4°C using degassed buffers as described before.<sup>110</sup>

**RNA analysis.** RNA was extracted and analysed by reverse transcriptase (RT) PCR as described before.<sup>110</sup> Primers for quantitative PCR (qPCR) are listed in Table 1. RT-qPCR data were standardized to *ACTB* as loading control. The percentage of *XBPI* splicing was calculated by dividing the signal for spliced *XBPI* mRNA by the sums of the signals for spliced and unspliced *XBPI* mRNAs. Band intensities were quantitated using ImageJ.

**Protein extraction and Western blotting.** Cells were washed three times with ice-cold PBS and lysed in RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, 0.1% (w/v) SDS] containing Roche complete protease inhibitors (cat. no. 11836153001, Roche Applied Science) and phosphatase inhibitors (cat. no. 04 906 837 001, Roche Applied Science) as described before.<sup>110</sup>

Proteins were separated by SDS-PAGE on 4-20% Criterion TGX Precast gels (cat. no. 567-1094, Bio-Rad Laboratories) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham HyBond<sup>TM</sup>-P, pore size 0.45  $\mu$ m, cat. no. RPN303F, GE Healthcare) by semi-dry electrotransfer in 0.1 M Tris, 0.192 M glycine, and 5% (v/v) methanol at 2 mA/cm<sup>2</sup> for 60-75 min. Membranes were then blocked for 1 h in 5% (w/v) skimmed milk powder in TBST [20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% (v/v) Tween-20] for antibodies against non-phosphorylated proteins and 5% BSA in TBST for antibodies against phosphorylated proteins. Incubations with antibodies were performed over night at 4°C with gentle agitation. Blots were washed three times with TBST and then probed with secondary antibody for 1 hour at room temperature. The rabbit anti-AKT, anti-phospho-S473-AKT, anti-phospho-S51-eIF2 $\alpha$ , anti-JNK and anti-phospho-JNK antibodies were used at a 1:1,000 dilution in TBST + 5% (w/v) BSA. The rabbit anti-eIF2 $\alpha$  antibody was used at a 1:500 dilution in TBST + 5% (w/v) skimmed milk powder. Membranes were developed with goat anti-rabbit-IgG (H+L)-horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:1,000 dilution in TBST + 5% (w/v) skimmed milk powder for 1 h at room temperature. The



mouse anti-CHOP antibody and anti- $\beta$ -actin antibodies were used at a 1:1,000 dilution in TBST + 5% (w/v) skimmed milk powder, and the mouse anti-GAPDH antibody at a 1:30,000 dilution in TBST + 5% (w/v) skimmed milk powder. These antibodies were developed with goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:20,000 dilution in TBST 5% (w/v) skimmed milk powder for 1 h at room temperature. The goat anti-HIF1 $\alpha$  antibody was used at a dilution of 1:500 in TBST + 5% (w/v) skimmed milk powder and developed with mouse anti-goat IgG (H+L)-HRP-conjugated antibody at a dilution of 1:30,000 in TBST + 5% (w/v) skimmed milk powder for 1 h at room temperature. To reprobe blots for detection of nonphosphorylated proteins, membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Loughborough, UK, cat. no. 21059) and blocked with 5% (w/v) skimmed milk powder in TBST.

For signal detection, Pierce ECL Western Blotting Substrate (cat. no. 32209) or Pierce ECL Plus Western Blotting Substrate (cat. no. 32132) from Thermo Fisher Scientific were used. Blots were exposed to CL-X Posure<sup>TM</sup> film (cat. no. 34091, Thermo Fisher Scientific). Exposure times were adjusted on the basis of previous exposures to obtain exposures in the linear range of the film. Films were scanned on a CanoScan LiDE 600F scanner (Canon) and saved as tif files. Bands were quantified using ImageJ exactly as described under the heading “Gels Submenu” on the ImageJ web site (<http://rsb.info.nih.gov/ij/docs/menus/analyze.html#plot>). Peak intensities for the experimental antibody were then divided by the peak intensities obtained with the antibody for the loading control in the corresponding lane to correct for differences in loading between individual lanes. All loading control-corrected peak intensities obtained for one Western blot were then expressed relative to the loading control-corrected peak intensity of the 0 h sample.

**Statistical analysis.** All data are presented as the average and standard error of three independently differentiated adipocyte cultures. Errors were propagated using the law of error

propagation for random, independent errors.<sup>111</sup> Statistical analyses were performed in GraphPad Prism 6.04. The statistical tests and corrections for multiple comparison used to analyse the data are described in detail in the figure legends.

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### Figure legends

**Figure 1. Adipocyte differentiation of 3T3-L1 and 3T3-F442A cells.** (A, B) Nile red fluorescence, (C, D) side scatter (SSC-H), and (E, F) forward scatter (FSC-H) of (A, C, E) 3T3-L1 and (B, D, F) 3T3-F442A cells before (0 d, grey lines) and 12 d after induction of adipocyte differentiation (black lines). The light grey lines represent the autofluorescence of cells differentiated for 12 d. Dot plots of the side scatter SSC-H versus the forward scatter SSC-H for 3T3-L1 and 3T3-F442A cells before and 12 d after differentiation are shown in Fig. S1. The mean Nile red fluorescence of preadipocytes is significantly different from the mean Nile red fluorescence of differentiated adipocytes in a one way analysis of variance (ANOVA) test with Dunnett's correction for multiple comparisons<sup>112, 113</sup> ( $p < 0.0001$  for both 3T3-L1 and 3T3-F442A cells).

**Figure 2. Palmitate does not induce CHOP protein expression or *XBPI* splicing in adipocytes.** (A, B) CHOP expression in *in vitro* differentiated (A) 3T3-F442A adipocytes and (B) 3T3-L1 adipocytes exposed to the indicated concentrations of palmitate complexed to BSA for 48 h. Relative (rel.) CHOP signals were corrected for the loading controls GAPDH or  $\beta$ -actin. The bar graphs show the average and standard error of three independent repeats. Differences are not statistically significant ( $p = 0.42$  for 3T3-F442A adipocytes and  $p = 0.10$

for 3T3-L1 adipocytes in a repeated measures ANOVA test that compares the treated samples to the untreated sample. Equal variabilities of the differences were assumed for the treated and untreated samples and Dunnett's correction for multiple comparisons<sup>112, 113</sup> was used). 1  $\mu$ M thapsigargin (Tg) was used as a positive control for induction of ER stress. Thapsigargin-treated samples were compared to untreated samples using a two-tailed, unpaired *t*-test. **(C, D)** *XBPI* splicing in *in vitro* differentiated (C) 3T3-F442A adipocytes and (D) 3T3-L1 adipocytes incubated for 48 h with the indicated concentrations of BSA-complexed palmitate. % splicing indicates the percentage of spliced *XBPI* mRNA, for which the average and standard error of three independent experiments are shown. Abbreviations: u – unspliced *XBPI* mRNA, s – spliced *XBPI* mRNA. **(E)** MTT assay on *in vitro* differentiated 3T3-F442A adipocytes incubated for 48 h with the indicated concentrations of BSA-complexed palmitate. A repeated measures ANOVA test was used to compare the treated samples to the untreated sample. Equal variabilities of the differences were assumed for the treated and untreated samples and Dunnett's correction for multiple comparisons<sup>112, 113</sup> was applied. **(F)** *XBPI* splicing in 3T3-F442A preadipocytes incubated for 12 h with the indicated concentrations of BSA-complexed palmitate. Abbreviations: \* -  $p < 0.05$ , \*\* -  $p < 0.0$ , \*\*\* -  $p < 0.001$ , and \*\*\*\* -  $p < 0.0001$ .

**Figure 3. Palmitate does not induce *BiP*, *CHOP*, or *ERDJ4* transcription in adipocytes.**

**(A, B)** *BiP* mRNA, **(C, D)** *CHOP* mRNA, and **(E, F)** *ERDJ4* mRNA levels in *in vitro* differentiated (A, C, E) 3T3-F442A and (B, D, F) 3T3-L1 adipocytes incubated for 48 h with the indicated concentrations of BSA-complexed palmitate. The differences in *BiP* mRNA ( $p = 0.10$  for 3T3-F442A adipocytes and  $p = 0.34$  for 3T3-L1 adipocytes), *CHOP* mRNA ( $p = 0.11$  for 3T3-F442A adipocytes and  $p = 0.41$  for 3T3-L1 adipocytes), and *ERDJ4* mRNA ( $p = 0.48$  for 3T3-F442A adipocytes and  $p = 0.41$  for 3T3-L1 adipocytes) levels in the untreated and palmitate treated samples are not statistically significant. A repeated measures ANOVA



test with Dunnett's correction for multiple comparisons<sup>112, 113</sup> and assuming equal variabilities of the differences was used to compare the palmitate-treated samples to the untreated sample. Thapsigargin-treated samples were compared to untreated samples using a two-tailed, unpaired *t*-test.

**Figure 4. Palmitate does not inhibit insulin signalling in 3T3-F442A adipocytes.** (A) Serum-starved 3T3-F442A adipocytes and (B) serum-starved undifferentiated 3T3-F442A cells were treated with the indicated concentrations of BSA-complexed palmitic acid for 48 h before stimulation with 100 nM insulin for 15 min. Phosphorylation of AKT at serine 473 and total AKT levels were determined by Western blotting.

**Figure 5. Cholesterol loading does not induce CHOP protein expression or *XBPI* splicing in adipocytes.** (A, B) CHOP protein levels and (C, D) *XBPI* splicing in *in vitro* differentiated (A, C) 3T3-F442A and (B, D) 3T3-L1 adipocytes incubated for 48 h with human acetylated LDL (AcLDL), AcLDL and 0.6  $\mu$ M of the ACAT inhibitor TMP-153, 0.6  $\mu$ M TMP-153, 1.0  $\mu$ M Tg, or left untreated ('-'). The average and standard error of three independent experiments are shown in the bar graphs. Differences in CHOP protein levels between the untreated sample and the samples treated with AcLDL, AcLDL and 0.6  $\mu$ M TMP-153, and 0.6  $\mu$ M TMP-153 are not statistically significant ( $p = 0.26$  for 3T3-F442A adipocytes and  $p = 0.35$  for 3T3-L1 adipocytes in a repeated measures ANOVA test with Dunnett's correction for multiple comparisons<sup>112, 113</sup> comparing the treated samples to the untreated samples and assuming equal variabilities of the differences). (E) *XBPI* splicing in untreated *in vitro* differentiated human THP-1 macrophages and macrophages incubated for 16 h with AcLDL, AcLDL + 0.6  $\mu$ M TMP-153, 0.6  $\mu$ M TMP-153, or 1.0  $\mu$ M Tg.

**Figure 6. Cholesterol loading does not induce *BiP* or *CHOP* transcription in adipocytes.** (A, B) *BiP* mRNA and (C, D) *CHOP* mRNA levels in *in vitro* differentiated (A, C) 3T3-F442A and (B, D) 3T3-L1 adipocytes incubated for 48 h with human acetylated LDL

(AcLDL), AcLDL and 0.6  $\mu$ M of the ACAT inhibitor TMP-153, 0.6  $\mu$ M TMP-153, 1.0  $\mu$ M Tg, or left untreated ('-'). The average and standard error of three independent experiments are shown. Differences are not statistically significant (*BiP* mRNA:  $p = 0.34$  for 3T3-F442A adipocytes and  $p = 0.11$  for 3T3-L1 adipocytes; *CHOP* mRNA:  $p = 0.09$  for 3T3-F442A adipocytes and  $p = 0.11$  for 3T3-L1 adipocytes).  $p$  values were obtained from a repeated measures ANOVA test comparing the samples treated with AcLDL, AcLDL and 0.6  $\mu$ M TMP-153, and 0.6  $\mu$ M TMP-153 to the untreated samples and assuming equal variabilities of the differences. Dunnett's correction for multiple comparisons<sup>112, 113</sup> was applied. Thapsigargin-treated samples were compared to untreated samples using a two-tailed, unpaired  $t$ -test.

**Figure 7. The proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  do not induce ER stress in adipocytes. (A)** MTT assay on *in vitro* differentiated 3T3-F442A adipocytes incubated for 24 h with the indicated concentrations of TNF- $\alpha$ . A repeated measures ANOVA test was used to compare the treated samples to the untreated sample. Equal variabilities of the differences were assumed for the treated and untreated samples and Dunnett's correction for multiple comparisons<sup>112, 113</sup> was applied. **(B)** *XBPI* splicing in *in vitro* differentiated 3T3-F442A adipocytes incubated for 24 h with the indicated concentrations of TNF- $\alpha$  or 1.0  $\mu$ M Tg. The average and standard error from three independent experiments are shown. **(C)** JNK phosphorylation in 3T3-F442A preadipocytes incubated for 30 min with 25 ng/ml TNF- $\alpha$ . **(D and E)** *XBPI* splicing in *in vitro* differentiated 3T3-F442A adipocytes incubated for 24 h with the indicated concentrations of (D) IL-6 and (E) IL-1 $\beta$ . The average and standard error of two independent experiments are shown. **(F)** JNK phosphorylation in 3T3-F442A preadipocytes incubated for the indicated times with 200 ng/ml IL-6 or 200 ng/ml IL-1 $\beta$ .

**Figure 8. Glucose starvation induces ER stress in adipocytes. (A)** CHOP protein levels in *in vitro* differentiated 3T3-F442A and 3T3-L1 adipocytes maintained for 24 h in the presence

of 4.5 g/l D-glucose ('+ Glucose') or without any glucose ('- Glucose').  $\beta$ -Actin was used as a loading control. **(B)** Quantitation of the Western blots shown in panel (A). **(C)** *XBPI* splicing in *in vitro* differentiated 3T3-F442A and 3T3-L1 adipocytes maintained for 24 h in the presence of 4.5 g/l D-glucose or without any glucose. Below the images of the agarose gels the intensity of the ethidium bromide fluorescence was plotted versus the migration distance of the PCR products. **(D)** Quantitation of *XBPI* splicing shown in panel (C). For both cell lines the average and standard error of three independent repeats are shown. **(E)** Steady-state mRNA levels of *CHOP*, *BiP*, *ERDJ4*, *EDEM1*, and *VEGFA* mRNAs in 3T3-F442A adipocytes maintained for 24 h in the presence of 4.5 g/l D-glucose or without any glucose. *p* values were obtained from two-tailed, unpaired *t*-tests.

**Figure 9. Hypoxia induces ER stress in adipocytes.** **(A-B)** Induction of HIF1 $\alpha$  and increased phosphorylation of eIF2 $\alpha$  at serine 51 in *in vitro* differentiated (A) 3T3-F442A and (B) 3T3-L1 adipocytes incubated for the indicated times under 0.5% (v/v) O<sub>2</sub>. **(C-D)** *XBPI* splicing in *in vitro* differentiated (C) 3T3-F442A and (D) 3T3-L1 adipocytes incubated for the indicated times under 0.5% (v/v) O<sub>2</sub>. Representative gels from three biological repeats are shown. **(E, F)** Steady-state *BiP* mRNA levels in *in vitro* differentiated 3T3-F442A (E) and 3T3-L1 (F) adipocytes incubated for the indicated times under 0.5% (v/v) O<sub>2</sub> were determined by RT-qPCR. *p* values were obtained from a repeated measures ANOVA test comparing the treated samples to the untreated samples and assuming equal variabilities of the differences. Dunnett's correction for multiple comparisons<sup>112, 113</sup> was employed.

828 **Table 1. Oligodeoxynucleotides.** The HUGO Gene Nomenclature Committee gene names  
 829 are given in brackets where these deviate from the commonly used gene names.

Name	Purpose	Sequence
H7961	<i>XBPI</i> PCR, forward primer	GATCCTGACGAGGTTCCAGA
H7962	<i>XBPI</i> PCR, reverse primer	ACAGGGTCCAACCTTGTCCAG
H7994	<i>ACTB</i> PCR and RT-qPCR, forward primer	AGCCATGTACGTAGCCATCC
H7995	<i>ACTB</i> PCR and RT-qPCR, reverse primer	CTCTCAGCTGTGGTGGTGAA
H8553	<i>BiP (HSPA5)</i> RT-qPCR, forward primer	TTCGTGTCTCCTCCTGAC
H8554	<i>BiP (HSPA5)</i> RT-qPCR, reverse primer	ACAGTGAACCTTCATCATGCC
H8660	<i>VEGFA</i> RT-qPCR, forward primer	AGAGCAACATCACCATGCAG
H8661	<i>VEGFA</i> RT-qPCR, reverse primer	TTTGACCCTTTCCTTTTCCT
H8736	<i>ERDJ4 (DNAJB9)</i> RT-qPCR, forward primer	CTGTGGCCCTGACTTGGGTT
H8737	<i>ERDJ4 (DNAJB9)</i> RT-qPCR, reverse primer	AGGGGCAAACAGCCAAAAGC
H8778	<i>CHOP</i> RT-qPCR, forward primer	TCTTGAGCCTAACACGTCGAT
H8779	<i>CHOP</i> RT-qPCR, reverse primer	CGTGGACCAGGTTCTGCTTT
H8796	<i>EDEMI</i> RT-qPCR, forward primer	TGGAAAGCTTCTTTCTCAGC
H8797	<i>EDEMI</i> RT-qPCR, reverse primer	ATTCCCGAAGACGTTTGTCC
H9106	<i>PERK</i> RT-qPCR, forward primer	CTCAAGTTTCTCTACTGTTCACTC
H9107	<i>PERK</i> RT-qPCR, reverse primer	GCTGTCTCAGAACCGTTTTCCT
H9110	<i>IRE1<math>\alpha</math></i> RT-qPCR, forward primer	GCGCAAATTCAGAACCTACAAAGG
H9111	<i>IRE1<math>\alpha</math></i> RT-qPCR, reverse primer	GGAAGCGGGAAGTGAAGTAGC



















